

## OBTAINING *Artemisia cina* POLYPLOIDY THROUGH PLANT GROWTH REGULATOR TREATMENT IN SHOOT CULTURE

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### ABSTRACT

*Artemisia cina* is a medicinal plant species which produces bioactive compound potential to anti-tumor, antifungal and antibacterial medicines. This study was aimed to obtain *A. cina* polyploid plants through a treatment of growth regulators in shoot culture. The shoot were treated in 1; 15; 2; and 3 mg L<sup>-1</sup> of 2,4-dichloro phenoxy acetic acid (2,4-D) combined with 1; 1.5; 2; 3 mg L<sup>-1</sup> of Benzyladenine (BA) for 28 days. Chromosome analysis showed that the highest polyploidy percentage, 23%, was reached in the treatment of 2 mg L<sup>-1</sup> of 2,4-D combined with 1 of BA mg L<sup>-1</sup>. The polyploidy level varied, comprising 2n=3x, 2n=4x, 2n=5x, 2n=6x, with the highest polyploidy level percentage, 28.57%, and it was attained in the tetraploid (2n=4x). Polyploid plants had larger leaves area, larger stomatal size, and higher chlorophyll content than diploid plants. However stomatal density of polyploidy plants was lower than that of in diploid plants.

Keywords: 2,4-D, *Artemisia cina*, BA, polyploidy

### INTRODUCTION

Malaria is a disease caused by *Plasmodium falciparum*, a pathogen which mostly attacks people in tropical areas including Indonesia. The treatment using *chloroquine* causes *plasmodium* to be resistant to this drug, therefore new drugs are required to solve the problem with this parasite's resistance (Aryanti, 2010). Artemisinin is a sesquiterpene lactone, a vastly active compound as anti-malarial and anti-tumor agents. The compound is based on artemisinin (called Artemisinin Combined Therapy) which is recommended by

World Health Organization (WHO) for malaria treatments in Africa (Aryanti, 2010). Artemisinin has been found in the leaves and shoots of *Artemisia annua*. Artemisinin content in every part of the plant is affected by environmental factors, for instance light intensity, altitude and cultivation condition (Ferreira and Janicks, 1996).

*Artemisia cina* (Asteraceae) is another species member of genus *Artemisia* which is used for tumor and malaria remedy (Aryanti *et al.*, 2001) as it contains artemisinin bioactive compound. Artemisinin existing in *Artemisia cina* is very low compared to *Artemisia annua*, hence compound escalation is required. Clone improvement through *in vitro* genetic manipulation is one of the potential strategies for artemisinin supply. One method to increase the artemisinin of *A. cina* is polyploidy induction.

Artificial polyploid is a technique to increase the chromosome quantity in plant. Polyploidy induction of *A. annua* is able to increase artemisinin production (Banyai *et al.*, 2010; Huang *et al.*, 2010). Artificial polyploid can be conducted by means of *in vitro* technique with *colchicine* in a culture medium and growing the explants in that medium in a certain period (Banyai *et al.*, 2010; Tepe *et al.*, 2009; Griesbach and Kamo, 1996; Jesus-Gonzales and Weathers, 2003; Huang *et al.*, 2010; Nilanthi *et al.*, 2009; Raza *et al.*, 2003; Xing *et al.*, 2009; Zhang *et al.*, 2007). In addition, Polyploidy can also be induced by using plant growth regulators such as auxin (2,4-D) and Cytokinin (BA) in culture medium. Fras and Maluszynska (2003) were successful to induce polyploidy in *Arabidopsis thaliana* using plant growth regulators. The same results was reported by Sulistyaningsih *et al.* (2006) in shallot. In order to produce polyploidy *A. cina*, we tried to induce

polyploidy *A. cina* using 2,4-D and BA and observed their growth characters.

## MATERIALS AND METHODS

### Explants Propagation in Shoot Culture

*Artemisia cina* plants used for shoot culture were from The Medicinal Plant Research and Development Center, Tawangmangu, Central Java, Indonesia. The use of shoot culture medium was MS medium complemented with 10 mg L<sup>-1</sup> of kinetin and 1 mg L<sup>-1</sup> of NAA (1-Naphthalaeneacetic acid). The explants used for shoot culture were *Artemisia cina* shoots cultured in vitro. Each explant consisted of 3 stem segments and those from segment 2 to segment 6 and was planted in MS medium containing 10 mg L<sup>-1</sup> of kinetin and 1 mg L<sup>-1</sup> of NAA. The explants were subcultured every 4 weeks by cutting off every 3 stem segments and replanting them in the fresh MS medium containing 10 mg L<sup>-1</sup> of kinetin and 1 mg L<sup>-1</sup> NAA.

### Polyploid Induction

The induction media used were MS media with plant growth regulators of 2,4-D (1.0; 1.5; 2.0 and 3 mg L<sup>-1</sup>) and BA (1.0; 1.5; 2.0 and 3 mg L<sup>-1</sup>). The identical explants were selected and displaced into polyploidy induction medium afterwards. The displacement of explant was conducted in two weeks after the last subculture at propagation stage. Polyploid induction *A. cina* using plant growth regulator was in the period of 28 days. After polyploidy induction, explants were transferred to the MS medium namely MS0 without growth regulators. Explants *A. cina* were also cultured in MS medium without 2,4-D and BA as sources of explants without polyploidy induction.

### Root Induction Stage

Explants of shoot culture were then displaced into root induction medium. The used medium was half MS medium containing 3 mg L<sup>-1</sup> of BA. The period of root induction was 28 days. Subsequently explants entered to acclimatization stage.

### Acclimatization Stage

After the explant formed a plantlet, it was then displaced into plastic pots with rice husk charcoal medium and incubated in the laboratory at room temperature for 14 days. When the

plantlets were transferred in the pots, the root tips were also cut for chromosome analysis. The N, P, K nutrition were given in the form of liquid fertilizer with a concentration of 2 mL L<sup>-1</sup>. After 14 days, the plantlets were then transferred to glasshouse for 14 days. Subsequently, the plantlet was displaced into polybag with diameter of 20 cm filled with a mixture of garden soil medium and manure with ratio of 1:1.

### Chromosome Analysis

After two-weeks growth in the glasshouse, the chromosome quantities of the plantlet were observed. One cm length of root tip of plants were cut. The root tips were treated with iced water at 4°C for 24 hours. Then the root tips of plant were fixed into Carnoy's solution/fixation solution (3 ethanol: 1 glacial acetic acid), and stored in the refrigerator at 4°C for at least for 1 hour. The root tips were rinsed with distilled water and hydrolized using HCl 1 N for 15 minutes at 60°C in waterbath. After that the root tips were stained with Fuchsin basic for 20 minutes and replaced in objective glass. The chromosom were observed using Olympus Microscope connected to Optilab photo-microscope.

### Analysis of Chlorophyll Content of Leaves

Analysis of chlorophyll content of leaves was analyzed by DMSO (Dimethyl sulfoxide) method (Hiscox and Israelstam, 1979) which had been modified. The completely unfolded leaves on the third segment were used as materials for chlorophyll analysis. As much as 0.04 gram of leaves sample was sliced into small pieces and added 5 mL DMSO. The solution was incubated in the dark at room temperature for 48 hours and later filtered with filter paper. The absorbance value were measured by using spectrophotometer (UV mini-1240, UV VIS Spectrophotometer, Shimadzu) at a wavelength of 649 and 665 nm (Wellburn, 1994). Based on the absorbance value, the chlorophyll content was calculated by using the following equations.

Calculation:

$$\text{Chlorophyll a} = (12:19 \times A_{665}) - (3:45 \times A_{649})$$

ug mL<sup>-1</sup>

$$\text{Chlorophyll b} = (21:99 \times A_{649}) - (5:32 \times A_{665})$$

ug mL<sup>-1</sup>

$$\text{Total Chlorophyll} = (18:54 \times A_{649}) + (6:87 \times A_{665})$$

ug mL<sup>-1</sup>

### Leaf Area Analysis

Leaf area measurement was done with a Leaf Area Meter Mark 2 type, Delta T, Burwell Cambridge, England. The leaves used in the measurement of leaf area were the ones that had been completely unfolded and were located in sections 3, 5, 8 and 10.

### Stomata Observation

Stomata characteristics were observed in the 4<sup>th</sup> segment. The epidermal surfaces of adaxial and abaxial leaves were brushed with non-colored nail polish and covered with transparent plastic tapes. The tapes were taken off and placed on object glass. The stomata length, width and density were observed using Olympus microscope with photomicroscope Optilab.

### Percentage of Polyploidy Obtained

The percentage of polyploidy obtained was counted by counting of polyploidy obtained in every treatment divided by total number of polyploidy occurrence and multiplied by 100%.

### Data Analysis

The data of chromosome analysis, data of survival rate and percentage of polyploidy plants were analyzed using descriptive analysis. The parametric data analysis using T-test at 5% level was conducted to determine the significance between the two treatments on the observed data of leaf area, stomatal size, stomatal density, and chlorophyll content.

## RESULTS AND DISCUSSION

### Polyploid Induction and Confirmation of Polyploidy by Cytological Analysis

The growth and morphogenesis of plants through in vitro was influenced by the balance and interaction of the growth regulators in explants. Auxin and cytokinin were broadly used in plant tissue culture placed into the growing medium. Auxin and cytokinin could induce cell division, chloroplast development, shoot and side

shoot growths. Benzyladenin (BA) was one of essential cytokinins for plants. BA could stimulate several things, including cell division, shoot emergence and shoot formation. 2,4-D was one of auxins which played an important role in callus formation. The chromosome analysis result showed that the combinations of 2,4-D and BA were able to induce polyploid of *A. cina* plant (Table 1 and Figure 1). The imbalanced of Cytokinin and auxin concentrations had the highest possibility to induce polyploid (Swartz, 1991). The highest polyploid percentage, approximately 23%, was obtained from the treatment using 2 mg L<sup>-1</sup> of 2,4-D combined with 1 mg L<sup>-1</sup> of BA (Table 1). The generated polyploid through combination treatment of 2,4-D and BA occurred due to the condition in which the growth regulators induced anaphase abnormalities during mitosis process (Swartz, 1991).

Ploidy level was obtained varied on  $2n=3x$ ,  $2n=4x$ ,  $2n=5x$ ,  $2n=6x$  (Figure 2). The high polyploidy level percentages were 28.57% for  $2n=4x=36$  and 23.81% for  $2n=3x=27$  (Figure 1). The varied polyploidy level occurred because microtubule threads formation of core spindle (spindle threads) were prevented, hence, the chromosome separation which marked the transition from metaphase to anaphase did not arise and lead to propagation of chromosomes without cell wall propagation. Therefore, the chromosomes remained in cytoplasm as spindle threads were not formed. The chromosomes, however, were able to separate from the centromere as well as the beginning of c-anaphase followed by the core wall formation. Thus, it ensued "restitution" of nucleus containing double chromosomes (Suryo, 1995). In this study the obtained modal number of chromosome obtained was 9. *A. cina* has unique chromosome numbers with both modal number of  $x=8$  and  $x=9$ . The normal plants of these species have chromosome numbers of diploid  $2n=2x=18$  (from modal  $x=9$ ) or polyploidy  $2n=4x=32$  (from modal  $x=8$ ) (Darlington and Wylie, 1955; de Padua *et al.*, 1999).

Table 1. Effects of Plant Growth Regulators on Polyploidy Induction of *A. cina*

Plant Growth Regulator		Survival Rate in vitro (%)	Number of plants survived in green house	Number of diploid plants	Number of polyploid plants	Percentage polyploid plants (%)
2,4-D (mg L <sup>-1</sup> )	BA (mg L <sup>-1</sup> )					
1	1	73.33	40	40	0	0
	1.5	73.33	20	20	0	0
	2	80.00	32	30	2	9
	3	80.00	18	17	1	5
1.5	1	60.00	14	14	0	0
	1.5	40.00	18	17	1	5
	2	60.00	16	16	0	0
	3	53.33	16	13	3	14
2	1	73.33	29	24	5	23
	1.5	46.67	7	6	1	5
	2	33.33	2	2	0	0
	3	46.67	6	5	1	5
3	1	33.33	10	7	3	14
	1.5	66.67	5	2	3	14
	2	73.33	14	13	1	5
	3	53.33	1	0	1	5

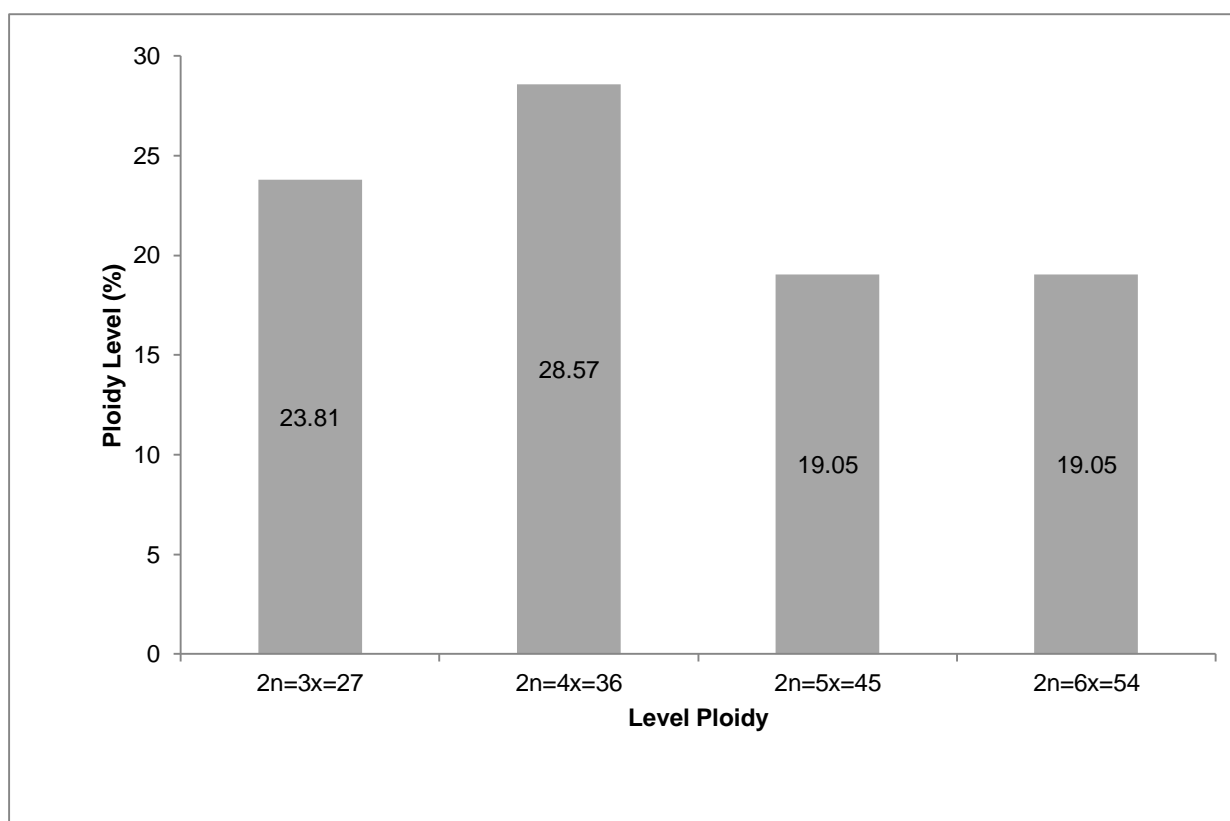


Figure 1. Percentage of polyploid level as induction result by means of growth regulators treatment

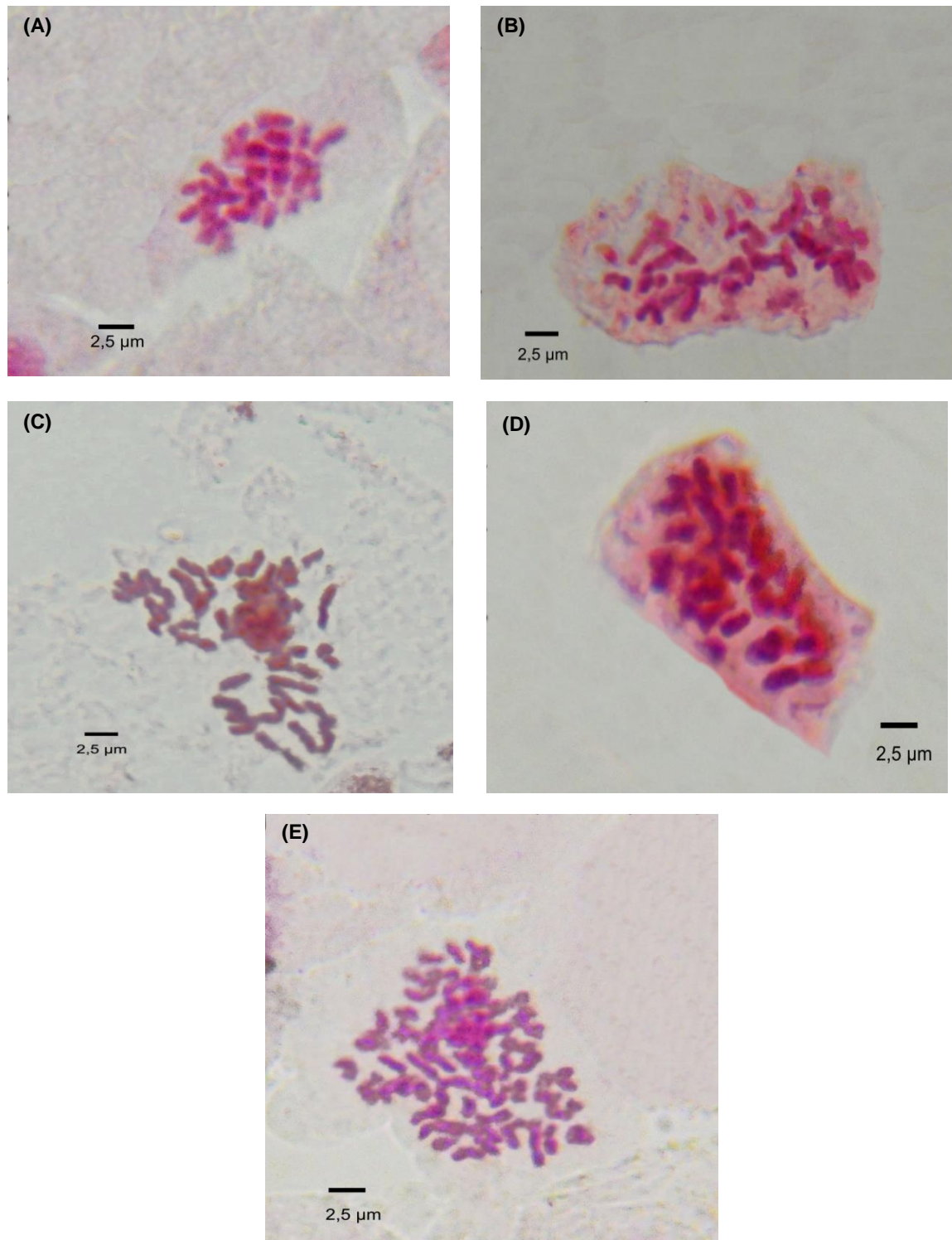


Figure 2. Chromosomes of root tip cells of *A. cina*, (A) chromosome number  $2n=2x=18$ ; (B)  $2n=3x=27$ ; (C)  $2n=4x=36$ ; (D)  $2n=5x=45$ ; (E)  $2n=6x=54$  (1000x magnification)

Table 2. Leaf characteristic of diploid and Polyploidy plants of *Artemisia cina*

Level Ploidy	Leaf Area (cm <sup>2</sup> )	Stomata Density (mm <sup>2</sup> )	Stomata Size (µm)		Chlorophyll Content (mg g <sup>-1</sup> )
			Length	Width	
Diploid (2n=2x=18)	20.75 ± 4.64	108.4 ± 4.91	22.15 ± 1.96	16.789 ± 0.80	1.97 ± 0.31
Triploid (2n=3x=27)	23.13 ± 6.64	90.44 ± 4.17*	27.38 ± 2.26 *	19.228 ± 2.91	2.44 ± 0.297*
Tetraploid (2n=4x=36)	28.31 ± 4.49*	88.69 ± 5.71*	30.02 ± 1.75*	22.73 ± 1.76*	2.355 ± 0.14*
Pentaploid (2n=5x=45)	20.65 ± 5.31	82.68 ± 8.89*	29.08 ± 0.98 *	22.025 ± 1.74*	2.318 ± 0.205
Heksaploid (2n=6x=54)	22.05 ± 8.577	83.40 ± 11.74*	30.36 ± 2.03*	21.23 ± 1.26*	2.283 ± 0.538

Remarks: An asterisk behind the number within the column indicate significant difference of mean (± SD) tested by T-test at p≤0.05

### Growth characterization of polyploidy plant

Growth characteristics in plants resulted from polyploid induction were evaluated and compared to diploid plants. Growth characteristics used to compare diploid and polyploidy plants were leaf area, stomata density, stomata size and leaf chlorophyll content. In Table 2, the leaf area on polyploidy plant was wider than that of diploid plants. Statistically only tetraploid's was different and significantly wider than that of diploid plants. Leaf stomata density was strongly influenced by ploidy level. The polyploidy plants had low stomata density. A low density of stomata on polyploidy plants was influenced by sizes of the stomata. The stomata size on polyploidy plants was greater than that of diploid plants. Greater stomata sizes may be used as consideration factors for polyploidy cytological determination.

Larger leaf size in polyploidy plants may indicate potentially higher biomass translating that higher quantity of desired compound existing in the leaves could be obtained (Kun-Hua *et al.*, 2010). On medicinal plants, the leaves often desirably became a source of active compounds, so that the increase in biomass associated with polyploidy plants were very attractive characteristics (Kun-Hua *et al.*, 2010). Higher yield or content of active compounds in polyploidy plants is essential for the extraction of natural products or drugs. Artemisinin is one active compound as an anti-malarial contained in *A. cina*. Considering the fact that artemisinin content in *A. cina* is still low, therefore induction of polyploidy plants of *A. cina* is an important stage to increase the production of artemisinin. Polyploidy plants have greater number of chromosomes than that of diploid plants, causing large the cell size and cell nucleus. The larger of

cell size will result to larger of plant size in general. Chlorophyll content in polyploid plants were higher than that of diploid plants, especially in triploid and tetraploid (Table 2). Polyploidy plants had larger leaf cells and higher amount of chlorophyll than diploid plants.

### CONCLUSION AND SUGGESTION

From the results, we concluded that 2,4-D growth regulators treatment combined with BA for 28 days was able to induce polyploidy *A. cina* plants in shoot culture. The treatment of 2 mg L<sup>-1</sup> of 2,4-D combined with 1 mgL<sup>-1</sup> of BA generated the highest polyploidy percentage (23 %). The polyploidy levels were varied, with the highest polyploidy level percentage was tetraploid (2n=4x=36). Leaf characteristics such as leaf area, stomatal size and chlorophyll content in polyploidy plants were significantly higher than those of diploid plants while stomatal density on polyploid plants was significantly lower than that of diploid plants. All polyploidy plants will be used as further studies in determination of artemisinin production. The result of this study was expected to give a contribution for the further studies of *A. cina* through shoot culture or genetic manipulation.

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## REFERENCES

- Aryanti. 2001. Artemisinin content variation of root hair and regeneration of *Artemisia China* berg ex poljakov as anti-cancer. Bogor Agricultural Institute Press. Bogor. p. 12-118.
- Aryanti. 2010. Production of artemisinin in shoot cultures of *Artemisia cina* irradiated callus. Indonesian J. Pharmacy 21 (1): 27-31.
- Banyai, W., R. Sangthong, N. Karaket, P. Inthima, M. Mii and K. Supaibulwatana. 2010. Overproduction of artemisinin in tetraploid *Artemisia annua* L. Plant Biotechnol. 27: 427-433.
- Darlington, C.D. and A.P. Wylie. 1955. Chromosome atlas of flowering plants. Allen and Unwin. London. pp. 519.
- dePadua, L.S., N. Bunyapraphatsara and R.H.M.J. Lemmens. 1999. Medicinal and poisonous plants 1. Plant Resources of South East Asia (PROSEA). PROSEA Foundation. Bogor. 12: 139-147.
- Ferreira, J.F.S. and J. Janick. 1996. Distribution of artemisinin in *Artemisia annua*. In: J. Janick (ed.) Progress in New Crops. ASHS Press. Arlington. Vatican. p. 579-584.
- Fras, A. and J. Maluszynska. 2003. Regeneration of diploid and tetraploid plants of *Arabidopsis Thaliana* via callus. Acta Biol. Cracov. Bot. 45 (2): 145-152.
- Griesbach, R.J. and K.K. Kamo. 1996. The effect of induced polyploidy on the flavonols of *Petunia 'Mitchell'*. Phytochemistry 42 (2): 361-363.
- Hiscox, J.D. and G.F. Israelstam. 1979. A method for the extraction of chlorophyll from leaf tissue without maceration. Can. J. Botany 57 (12): 1332-1334.
- Huang, H.P., S.L. Gao, L.L. Chen and K.H. Wei. 2010. *In vitro* tetraploid and generation of tetraploids from mixoploids in *Dioscorea zingiberensis*. Pharmacogn. Mag. 6 (21): 51-56.
- Jesus-Gonzales, L.D. and P.J. Weathers. 2003. Tetraploid *Artemisia annua* hairy roots produce more artemisinin than diploids. Plant Cell Rep. 21: 809-813.
- Kun-Hua, W., G. Shan-Lin and H. He-Ping. 2010. Tissue culture and generation auto-tetraploid plants of *Sophora flavescens* Aiton. Pharmacogn. Mag. 6 (24): 286-292.
- Nilanthi, D., X.L. Chen, F.C. Zhao, Y.S. Yang and H. Wu. 2009. Induction of tetraploids from petiole explants through colchicine treatments in *Echinacea purpurea* L. J. Biomed. Biotechnol. p. 1-7. doi:10.1155/2009/343485.
- Raza, H., M.J. Jaskani, M.M. Khan and T.A. Malik. 2003. *In vitro* induction of polyploids in Watermelon and estimation based on DNA content. Int. J. Agri. Biol. 5 (3): 298-302.
- Sulistyaningsih, E., Y. Aoyagi and Y. Tashiro. 2006. Flower bud culture of Shallot (*Allium cepa* L. Aggregatum group) with cytogenetic analysis of resulting gynogenic plants and somaclones. Plant Cell Tiss. Org. 86: 249-255.
- Suryo. 1995. Cytogenetic. Gadjah Mada University Press. Yogyakarta. pp. 446.
- Swartz, H.J. 1991. Post culture behavior: Genetic and epigenetic effects and related problems. In: Debergh, P.C. and R.H. Zimmerman (eds.) Micropropagation: technology and application. Springer Netherlands. p. 95-121.
- Tepe, S., S. Ellialtioglu and N. Yenice. 2009. Obtaining poliploid mint (*Mentha longifolia* L.) plants with *in vitro* colchicine treatment. Fac. of Agriculture and Fac. of Science. Ankara University and Menderes University. Turkey. p. 1-4.
- Wellburn, A.R. 1994. The spectral determination of chlorophylls a and b, as well as total carotenoids, using various solvents with spectrophotometers of different resolution. J. Plant Physiol. 144 (3): 307-313.
- Xing, S.H., X.B. Guo, Q. Wang, Q.F. Pan, Y.S. Tian, P. Liu, J.Y. Zhao, G.F. Wang, X.F. Sun and K.X. Tang. 2009. Induction and flow cytometry identification of tetraploids from seed-derived explants through colchicine treatments in *Catharanthus roseus* (L.) G. don. J. Biomed. Biotechnol. p. 1-10. doi:10.1155/2011/793198.
- Zhang, Z., H. Dai, M. Xiao and X. Liu. 2007. *In vitro* induction of tetraploids in *Phlox subulata* L. Euphytica. 159: 59-65.